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Secreted Protein Acidic and Rich in Cysteine (SPARC) Mediates Metastatic Dormancy of Prostate Cancer in Bone*[S]

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Sambad Sharma[‡], Fei Xing[‡], Yin Liu[‡], Kerui Wu[‡], Neveen Said[§], Radhika Pochampally[¶], Yusuke Shiozawa[‡], Hui-Kuan Lin[‡], K. C. Balaji[‖], and Kounosuke Watabe[‡1]

From the Departments of [‡]Cancer Biology, [§]Pathology, and [‖]Urology, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157 and the [¶]Department of Biochemistry and Cancer Institute, University of Mississippi Medical Center, Jackson, Mississippi 39216

Prostate cancer is known to frequently recur in bone; however, how dormant cells switch its phenotype leading to recurrent tumor remains poorly understood. We have isolated two syngeneic cell lines (indolent and aggressive) through *in vivo* selection by implanting PC3mm stem-like cells into tibial bones. We found that indolent cells retained the dormant phenotype, whereas aggressive cells grew rapidly in bone *in vivo*, and the growth rates of both cells in culture were similar, suggesting a role of the tumor microenvironment in the regulation of dormancy and recurrence. Indolent cells were found to secrete a high level of secreted protein acidic and rich in cysteine (SPARC), which significantly stimulated the expression of BMP7 in bone marrow stromal cells. The secreted BMP7 then kept cancer cells in a dormant state by inducing senescence, reducing "stemness," and activating dormancy-associated p38 MAPK signaling and p21 expression in cancer cells. Importantly, we found that SPARC was epigenetically silenced in aggressive cells by promoter methylation, but 5-azacytidine treatment reactivated the expression. Furthermore, high SPARC promoter methylation negatively correlated with disease-free survival of prostate cancer patients. We also found that the COX2 inhibitor NS398 down-regulated DNMTs and increased expression of SPARC, which led to tumor growth suppression in bone *in vivo*. These findings suggest that SPARC plays a key role in maintaining the dormancy of prostate cancer cells in the bone microenvironment.

Prostate carcinoma at an early stage is generally treated with surgical resection or radiotherapy with or without combination of androgen deprivation therapy (1–3). However, patients often develop fatal recurrent disease months or years after treatment of the primary tumor. The culprits for the recurrent disease are the small number of residual cells that are disseminated from the primary tumor prior to treatment (4). Even patients with asymptomatic disease or no evidence of primary disease progression are known to often harbor cancer cells at distant organs such as bone, and they can be isolated from the bone marrow aspirate (5). These cells include quiescent cancer stem cells (CSCs)[2] and may reacquire clonogenic growth in a favorable environment and cause recurrent disease, which is evident in 20–50% of patients who were treated for localized primary disease (6).

Metastatic dormancy at a distant site is known to be determined by intrinsic molecular characteristics of the cell as well as extrinsic cues from the microenvironment. Dormancy of micrometastasis and cellular dormancy have been described as the adapted modes of dormant survival in a distant environment (7–10). The ratio of p38 to Erk activation is considered to be one of the molecular indications that dictate the fate of cancer cells. A high ratio of activated p38 to Erk signals for inhibition of cell proliferation or cellular dormancy, whereas a low ratio reverts the phenotype to the proliferative state (8, 11, 12). Recently, it was also demonstrated that dormant cells are reprogrammed by epigenetic regulation that leads to a quiescence state (13). In addition, interaction between tumor cells and the stroma, angiogenesis, and immune surveillance of cancer cells are also known to regulate dormancy and recurrence (8, 14).

The lack of an established model for dormancy has been a major hurdle for research advancement in this field. Although several *in vitro* models have been described previously, *in vivo* working models for dormant and recurrent growth have yet to be developed. Recent attempts have characterized syngeneic head and neck squamous cell carcinoma cells (T-Hep and D-Hep) or a pair of breast cancer cell lines (D2.OR and D2A1 cells) that recapitulates dormant growth *in vivo* (13, 15–17). However, there is still no appropriate *in vivo* model that mimics dormancy and recurrence for prostate cancer, especially one that replicates the phenomenon of bone recurrence in patients. In an approach to identify dormant prostate cancer cells in bone, we isolated a pair of cell lines from the bones of mice that showed either aggressive growth or indolent disease when their CSCs were injected in the tibiae. Gene profiling of the paired cell lines revealed the role of secreted protein acidic and rich in cysteine (SPARC), also known as Osteonectin, in dormancy of tumor cells in bone. Our results suggest that SPARC maintains

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[S] This article contains supplemental Figs. S1–S5 and Table S1.

[1] To whom correspondence should be addressed: Dept. of Cancer Biology, Wake Forest University School of Medicine, Winston-Salem, NC 27157. Tel.: 336-716-0231; Fax: 336-716-0255; E-mail: kwatabe@wakehealth.edu.

[2] The abbreviations used are: CSC, cancer stem cell; SPARC, secreted protein acidic and rich in cysteine; BMP, bone morphogenetic protein; BMSC, bone marrow stromal cell(s); CM, conditioned medium/media; BMPR, bone morphogenetic protein receptor; TCGA, The Cancer Genome Atlas; DNMTs, DNA methyltransferases; hBMSC, human bone marrow stromal cells; rSPARC, recombinant SPARC.

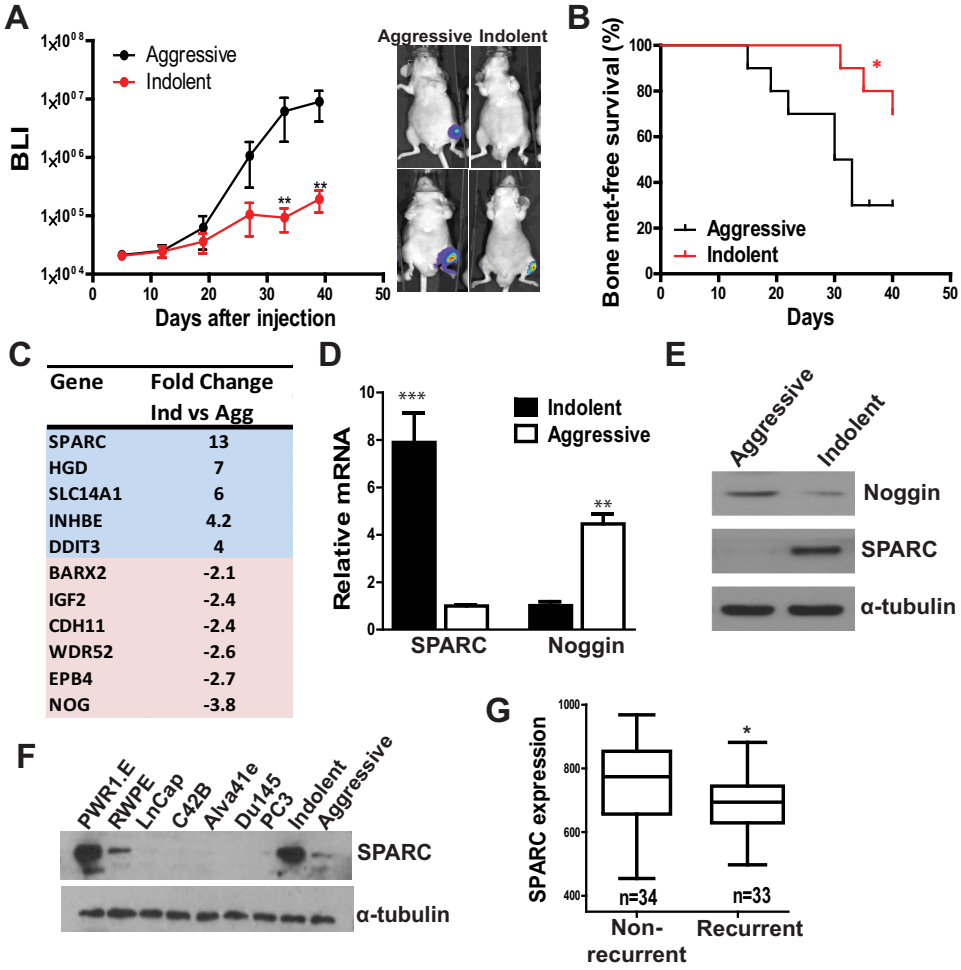

FIGURE 1. **Establishing model cell lines for dormancy recurrence.** *A*, indolent and aggressive cells were injected into the tibial bone of mice ($n = 8$), followed by examination of tumor growth by bioluminescence for 6 weeks. *Right panel*, representative pictures of aggressive and indolent cell growth in the tibiae of mice on day 40. **, $p < 0.01$ *versus* indolent. *BLI*, bioluminescence imaging. *B*, indolent and aggressive cells were injected via the intracardiac route, followed by examination of bone metastasis-free survival of the mice by bioluminescence ($n = 10$/group). *, $p = 0.0277$ by log-rank test. *C*, indolent (*Ind*) and aggressive (*Agg*) cells were subjected to comprehensive gene expression analysis by using Affymetrix microarray 2.0. The five most significantly up-regulated and six down-regulated genes are shown. *D* and *E*, SPARC and Noggin expression in indolent and aggressive cells was examined by quantitative RT-PCR (*D*) and Western blotting (*E*). *F*, SPARC expression was examined in normal and various prostate cancer cell lines by Western blotting. *G*, analysis of a Gene Expression Omnibus dataset for SPARC expression in patients with or without recurrent disease (GSE25136). 15% outliers were removed from the analysis. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$.

the dormant state of cancer cells by stimulating the secretion of bone morphogenetic protein 7 (BMP7), a TGF-β family member protein, from the bone stroma. SPARC was also found to be epigenetically controlled, and a COX2 inhibitor effectively suppressed tumor growth in bone by up-regulating SPARC, suggesting this microenvironmental cue as a potential therapeutic target for recurrent disease.

Results

Isolation of Paired Prostate Cell Lines as a Model for Dormancy and Recurrent Growth in Bone—To establish a model for dormancy and recurrent growth of prostate cancer, we first prepared CSCs from the PC3mm cell line using the defined cell surface markers $CD24^{low}/CD44^{high}/CD133^{high}$, as reported previously (18). CSCs were then implanted into mouse tibial bones with a dose by which ~50% of tibiae developed overt tumors after 1 month. The cells were then isolated from the bone with or without overt tumor growth, followed by colony expansion of each cell in culture (supplemental Fig. S1*A*). The cells derived from bone with overt growth or no growth were designated as "aggressive" and "indolent" cells, respectively. When the cells were reinjected into the tibial bone of mice, indolent cells grew significantly slower with a long lag time compared with aggressive cells (Fig. 1*A*). Furthermore, indolent cells also showed a decreased ability to colonize in bone when injected intracardially (Fig. 1*B*). However, in contrast to their striking difference in *in vivo* growth in bone, aggressive and indolent cells showed no difference in *in vitro* cell proliferation, invasion, migration, CSC population, and self-renewal abilities (supplemental Fig. 1, *B–F*), suggesting a role of the bone microenvironment in differential *in vivo* growth. We then performed Affymetrix microarray profiling to analyze differentially expressed genes between indolent and aggressive cells. As shown in Fig. 1*C*, SPARC and Noggin were most significantly overexpressed in indolent cells and aggressive cells, respectively. Both SPARC and Noggin are secretory proteins that are known to be present in the bone (19–22), which provides clues that the bone environment may play a pivotal role in the differ-

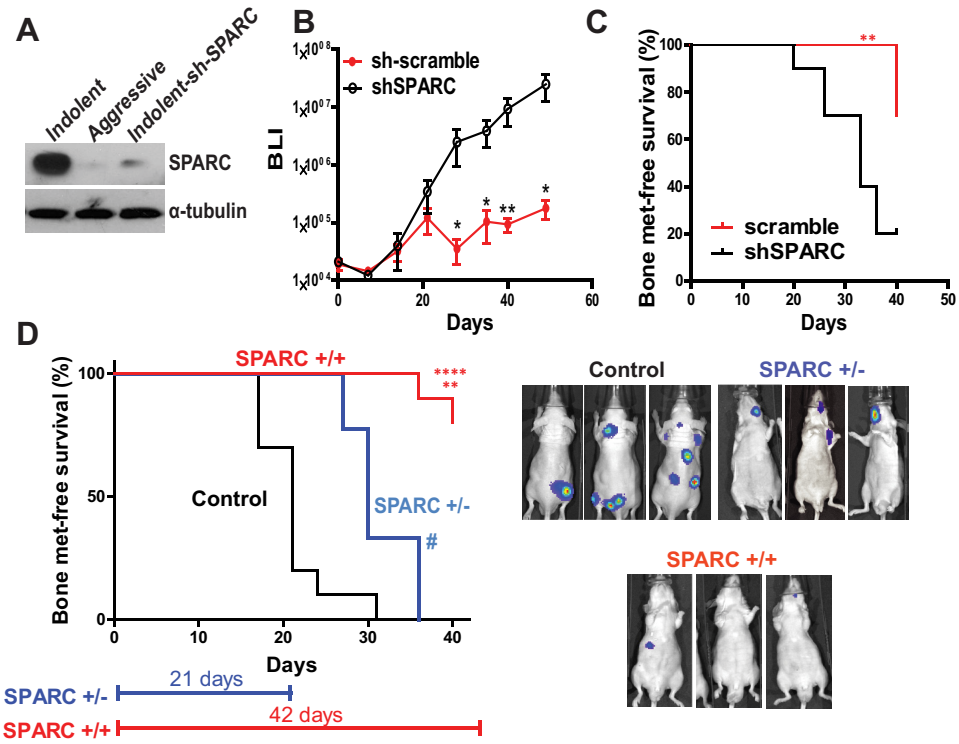

FIGURE 2. **SPARC plays a critical role in dormancy and recurrence.** *A*, SPARC knockdown by shRNA in indolent cells was verified by Western blotting. *B*, tumor growth was observed after intratibial injection of indolent-sh-SPARC or sh-Scramble cells by bioluminescence ($n = 10$/group). *, $p < 0.05$ *versus* scramble; **, $p < 0.01$ *versus* scramble. *BLI*, bioluminescence imaging. *C*, indolent-sh-SPARC or sh-Scramble cells were implanted into nude mice intracardially ($n = 10$/group), and bone metastasis-free survival was examined by bioluminescence. Indolent-Scramble *versus* indolent-sh-SPARC: **, $p = 0.0032$. *D*, aggressive cells were implanted into nude mice via intracardiac injection ($n = 10$/group), and bone metastasis-free survival was examined in the following three groups of animals: rSPARC (200 μg/kg) was administered by tail vein injection twice a week until day 42 (*red line*, +/+); rSPARC was administered until day 21, followed by withdrawal of rSPARC injection (*blue line*, ±); and a control group (*black line*) without administration of rSPARC. *Right panel*, representative images of mice from each group. Control *versus* SPARC+/+: ****, $p < 0.0001$; control *versus* SPARC ±: #, $p = 0.0117$; SPARC ± *versus* SPARC+/+: **, $p = 0.0099$ by log-rank test.

ential growth of indolent and aggressive cells *in vivo*. We further verified that the expression of Noggin was increased whereas the SPARC level was decreased in aggressive cells (Fig. 1, *D* and *E*). In addition, we found that SPARC expression in indolent cells was significantly higher compared with a series of prostate cancer cell lines and similar to that of normal immortalized prostate cells (Fig. 1*F*). Interestingly, when we analyzed a Gene Expression Omnibus database for expression of SPARC in a retrospective cohort of patients, we found that SPARC expression was significantly up-regulated in patients with no recurrence status for at least 5 years after radical prostatectomy (Fig. 1*G*) (23). These results strongly suggest that SPARC and Noggin play critical roles in the dormancy of prostate cancer.

SPARC Induces Dormancy in Vivo—SPARC is a matrix-associated protein, and it has been reported to be involved in cell cycle regulation, whereas Noggin is an inhibitor of BMPs (24–26). To further clarify the role of SPARC, the gene was silenced by introducing shRNA with a lentiviral expression system to indolent cells (Fig. 2*A*). Silencing SPARC in indolent cells or treating aggressive cells with recombinant SPARC did not confer any *in vitro* proliferative advantage to these cells (data not shown). We then transplanted indolent cells with or without expression of shRNA into tibial bone in nude mice. As shown in Figs. 2*B*, we found that knockdown of SPARC significantly stimulated the growth of tumor in bone, whereas scrambled shRNA did not affect the growth (Fig. 2*B*). Similarly, when tumor cells were transplanted via the intracardiac route, indolent cells that were silenced for SPARC expression showed a significant decrease in bone metastasis-free survival (Fig. 2*C*). To further examine the effect of SPARC on dormancy, we performed a recurrence assay *in vivo* by injecting aggressive cells into the nude mice via the intracardiac route, followed by administration of recombinant SPARC through intravenous injection every 3 days (Fig. 2*D*). We found that SPARC significantly delayed the incidence of bone metastasis. Importantly, withdrawal of SPARC injection after 3 weeks nullified the suppressive effect and significantly accelerated the onset of bone metastasis. These results suggest that SPARC leads to dormant survival of aggressive cells in bone and that the withdrawal of SPARC confers recurrent growth ability to cancer cells residing in bone (Fig. 2*D*).

SPARC Enhances Paracrine Dormancy Signaling from the Bone Stroma—The striking growth difference of indolent cells between *in vitro* and *in vivo* settings and the effect of recombinant SPARC *in vivo* strongly suggest that the secreted SPARC from indolent cells affects bone environmental cells to induce growth-suppressive effects on tumor cells. To test this hypothesis, we first cultured indolent and aggressive cells in a transwell plate with various cell types known to reside in bone. We found a significant reduction in the growth of indolent compared with aggressive cells when co-cultured with either BMSC or the HS5 bone stromal cell line, suggesting that secreted factor(s) from indolent cells induce an inhibitory response from bone stromal cells (Figs. 3, *A* and *B*, and supplemental Fig. S2*A*). Treatment of

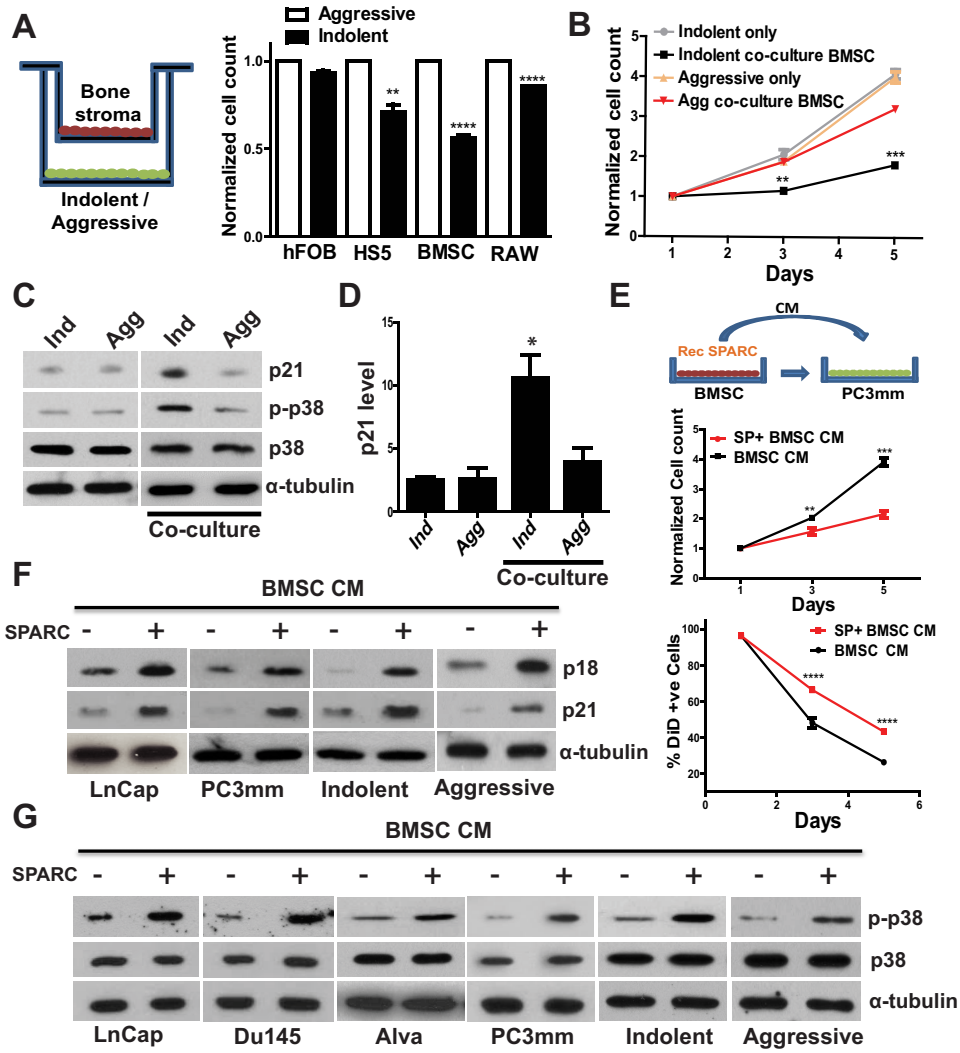

FIGURE 3. **SPARC-induced secretory factor from bone stroma activates p38 MAPK pathway.** *A*, indolent or aggressive cells were co-cultured with various bone stromal cells in a transwell, as shown in the *left panel*, followed by a cell proliferation assay of cancer cells in the lower chamber on day 5. *B*, indolent or aggressive cells were co-cultured with BMSC in a transwell plate, followed by quantifying cell proliferation of cancer cells. *C*, expression of p21, total p-38, and phosphorylated-p-38 was examined in indolent (*Ind*) and aggressive (*Agg*) cells with or without co-culture with BMSC for 3 days. *D*, p21 expression was examined by quantitative RT-PCR for *C*. *E*, *top panel*, BMSC were treated with or without rSPARC (200 ng/ml) for 24 h, and then the medium was replaced with fresh medium (DMEM) and incubated for 24 h to generate CM. *BMSC CM* and *SP+ BMSC CM* represent rSPARC-untreated or -treated BMSC CM, respectively. *Center* and *bottom panels*, PC3mm cells were seeded in a 96-well plate and treated with CM from SPARC-treated or -untreated BMSC as mentioned above, followed by examination of cell proliferation by (3-(4,5-dimethylthiazol-2 yl)-5-(3-carboxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (*center panel*) and DiD membrane labeling assay (*bottom panel*) on days 1, 3, and 5. *F*, CM generated from BMSC as shown in *E*, *top panel*, were used to treat prostate cancer cells (LnCaP, PC3mm, indolent, and aggressive) for 24 h, and p21 and p18 expression was examined by Western blotting. *G*, CM generated as shown in *E*, *top panel*, were used to treat a series of prostate cancer cells lines (as indicated) for 24 h, and phosphorylated as well as total p38 levels were examined by Western blotting. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$.

tumor cells with conditioned medium (CM) generated from BMSC or HS5 cells alone showed no differential proliferative effect (supplemental Fig. S2, *B* and *C*), suggesting a need of stroma-cancer cell interaction to exert the tumor-suppressive effect of stromal cells. Furthermore, we found that the transwell co-culture of indolent cells activated the dormancy-associated p38 pathway and also induced the p21 cell cycle inhibitor (Fig. 3, *C* and *D*). To examine whether the inhibitory response is indeed induced by SPARC, we first generated CM by treating BMSC with or without recombinant SPARC (as outlined in Fig. 3*E*, *top panel*) and treated cancer cells with the CM to examine cell proliferation by (3-(4,5-dimethylthiazol-2 yl)-5-(3-carboxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, or label retention assay (Fig. 3*E*, *center* and *bottom panels*). We found that the CM generated from SPARC-treated BMSC significantly lowered cell proliferation of PC3mm cells. Furthermore, CM of SPARC-treated BMSC activated p38-MAPK signaling and up-regulated expression of its downstream cell cycle inhibitors p21 and p18 in a panel of prostate cancer cells (Fig. 3, *F* and *G*). We also observed a significant decrease in cell proliferation and increase in p21 expression when CM were generated from BMSC or HS5 cells that were pretreated with aggressive or indolent cell-derived CM (supplemental Fig. S2, *D* and *E*). In addition, we also verified that SPARC secreted by indolent cells was significantly higher than that secreted by bone-residing cells (supplemental Fig. S2 *F*). These results strongly suggest that indolent cells secrete SPARC, which then stimulates BMSC to release factor(s) that, in turn, activate dormancy signaling in cancer cells in bone.

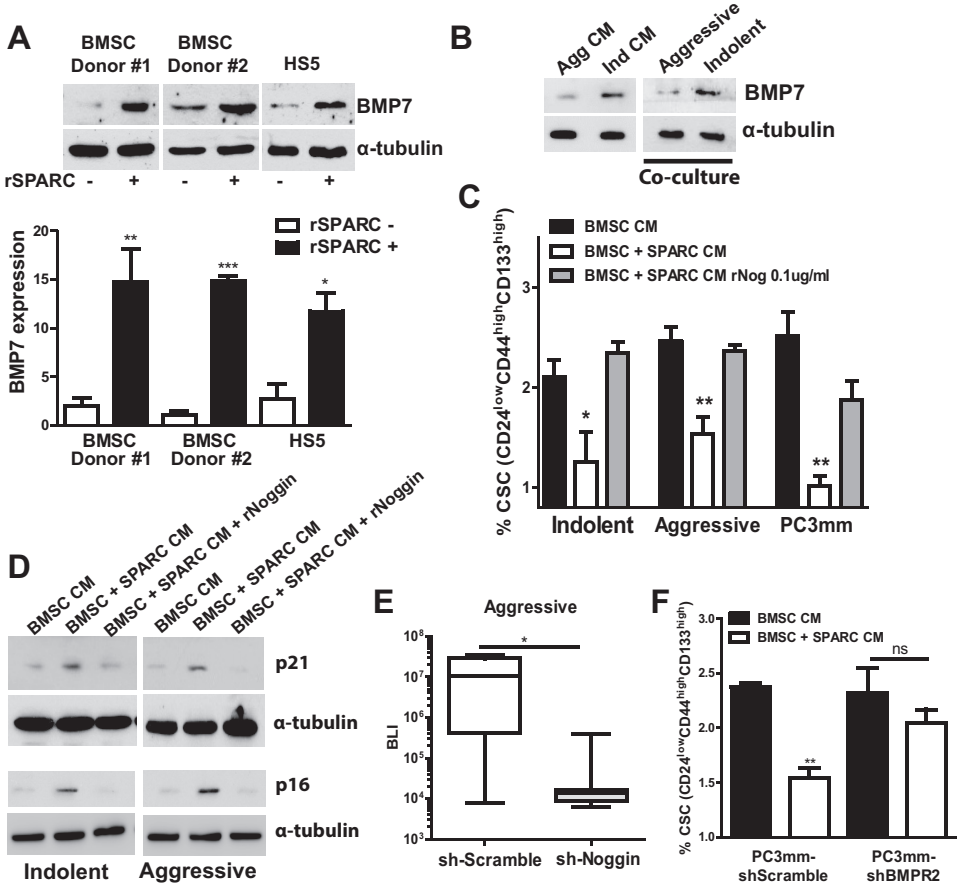

FIGURE 4. **SPARC up-regulates BMP7 from BMSC.** *A*, BMSC from two different donors (BMSC#1 and BMSC#2) and the HS5 cell line were treated with rSPARC (200 ng/ml) for 24 h, and BMP7 expression was examined by Western blotting and quantitative RT-PCR. *B*, BMSC#1 was treated with CM generated from indolent (*Ind*) or aggressive (*Agg*) cells for 24 h, followed by examination of BMP7 expression by Western blotting (*left panel*). BMP7 expression was also examined in BMSC co-cultured in a transwell with indolent or aggressive cells for 24 h (*right panel*). *C*, indolent, aggressive, and PC3mm cells were treated with the indicated CM for 48 h, followed by examination of the CSC population ($CD24^{low}/CD44^{high}/CD133^{high}$) by FACS. *D*, aggressive and indolent cells were treated with the indicated CM for 48 h, followed by examination of p21 (*top panel*) and p16 levels (*bottom panel*) by Western blotting. *E*, aggressive-sh-Noggin or -Scramble cells were implanted into the tibial bone of mice ($n = 6$/group), and the tumor growth was observed on day 28. *, $p < 0.05$ *versus* scramble. *F*, PC3mm-shScramble or PC3mm-shBMPR2 cells were treated with CM (as indicated) for 48 h, followed by examining the CSC marker-positive cells by FACS. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$; *ns*, not significant.

SPARC Up-regulates BMP7 Expression and Secretion from Bone Stroma—We reported previously that stromal expression of BMP7 induced dormancy by reducing "stemness" and inducing reversible senescence of prostate cancer cells in bone (18). Therefore, we wondered whether it was possible that SPARC up-regulates BMP7 expression in bone stromal cells. When we treated human primary BMSC that were isolated from two different donors or the bone stromal cell line HS5 with recombinant SPARC, we found that BMP7 was indeed significantly up-regulated (Fig. 4*A*). Furthermore, BMP7 was also up-regulated in BMSC that were treated with CM derived from indolent cells compared with CM from aggressive cells or from indolent-sh-SPARC cells (Fig. 4*B* and supplemental Fig. S3*A*). BMP7 was also found to be augmented in BMSC when co-cultured with indolent cells in transwell culture, and knockdown of SPARC in indolent cells significantly reduced BMP7 expression from BSMC in transwell culture (Fig. 4*B* and supplemental Fig. S3*A*). To further verify whether SPARC-induced BMP7 increases the dormancy phenotype of cancer cells, we generated CM by treating BMSC with recombinant SPARC and incubated cancer cells with the CM for 48 h. We found that the CM from SPARC-treated BMSC significantly decreased the stem cell population and sphere-forming ability and also increased senescence in cancer cells (Fig. 4*C* and supplemental Fig. S3, *B* and *C*). Furthermore, SPARC-treated BMSC CM increased p16 and p21 expression in cancer cells (Fig. 4*D*). Importantly, when recombinant Noggin, a competitive inhibitor of BMPs, was added to the SPARC-treated BMSC CM, the inhibitory effect of SPARC-treated BMSC CM was rescued (Fig. 4, *C* and *D*), further verifying that the dormancy phenotype is indeed mediated by BMP7. In addition, silencing Noggin expression in aggressive cells significantly inhibited its ability to grow in bone *in vivo* (Fig. 4*E* and supplemental Fig. S3*D*), suggesting that the absence of Noggin enhanced stromal BMP7-mediated growth suppression of tumor cells. The activation of bone morphogenetic protein receptors (BMPRs) by BMP7 is known to trigger the downstream signaling cascade (27). We have previously shown that BMPR2 is one of the major receptors associated with dormancy of prostate cancer cells and that BMP7 selectively binds this receptor (18). Therefore, to examine whether the decrease in cancer stemness is mediated through BMPR2, we knocked down BMPR2 in PC3mm

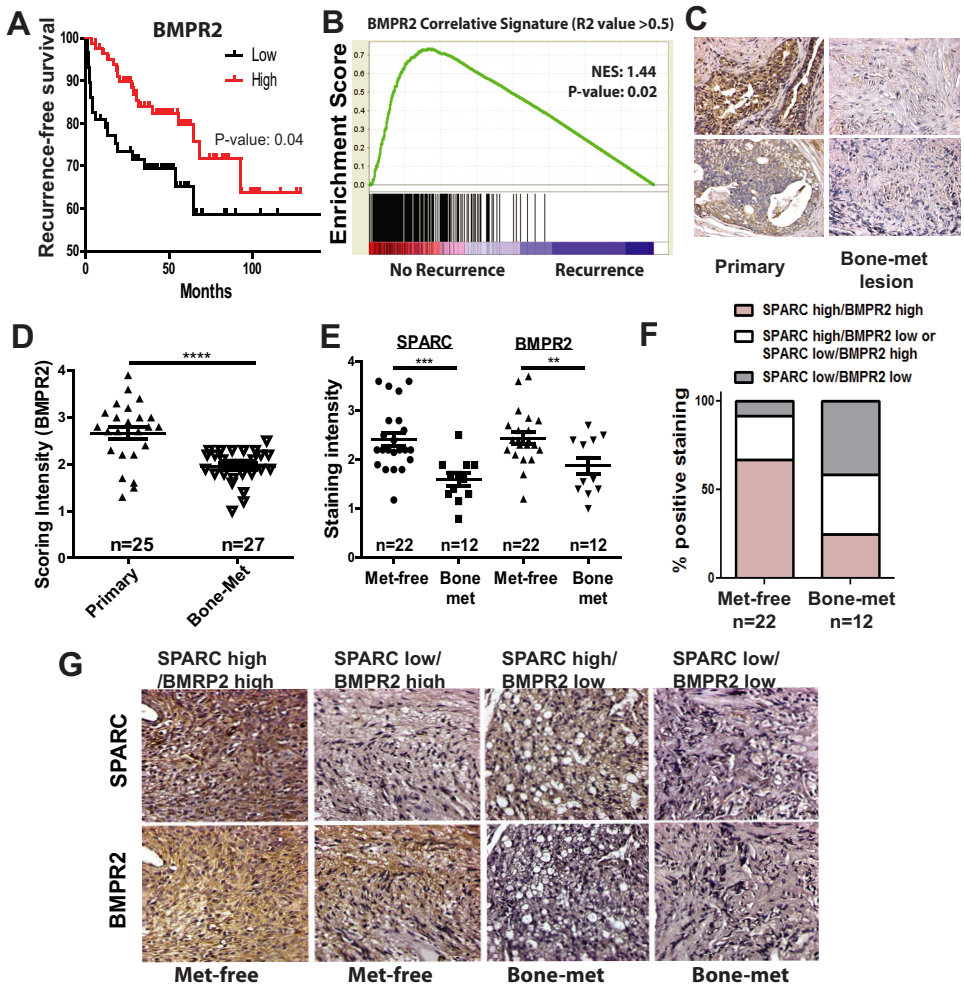

FIGURE 5. **SPARC and BMPR2 expression is negatively correlated with bone metastasis.** *A*, patients were stratified based on BMPR2 gene expression, and recurrence-free survival was examined using the GSE21034 database ($n = 140$). *B*, BMPR2 correlative signature ($n = 378$, $R^2 > 0.5$) was established using TCGA database for prostate cancer, and enrichment of the signature was examined in patients with or without recurrent disease (GSE21034). *NES*; normalized enrichment score. *C*, representative images of BMPR2 staining in primary tumor tissue and bone metastatic lesion. *D*, quantitation of BMPR2 staining in primary tumor tissue and bone metastatic lesion. *E*, SPARC and BMPR2 expression was examined in a primary tumor of patients with or without bone metastasis by immunohistochemistry, and the staining intensity was quantified. Consecutive sections of formalin-fixed, paraffin-embedded samples were used to stain SPARC and BMPR2 for each patient. *Met*, metastasis. *F*, the stained samples were divided into three groups (positive for either SPARC or BMPR2 (−/+ and ±, respectively), positive for both SPARC and BMPR2, and negative for both BMPR2 and SPARC expression based on their staining intensity), and the number of samples in each group was plotted as the percentage of total stained samples from either metastasis-free (localized disease) or bone metastatic primary tumor. *G*, representative images for SPARC and BMPR2 staining. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$.

cells that were then treated with CM generated from BMSC with or without SPARC treatment. As shown in Fig. 4*F*, the treatment of PC3mm cells with the CM significantly reduced the CSC population, whereas knockdown of BMPR2 blocked this suppressive effect of CM, indicating that the decrease in stemness is signaled through BMPR2. These results strongly suggest that stromal education by tumor-secreted SPARC up-regulates BMP7, which binds the BMPR2 receptor on the cancer cell to promote the dormancy phenotype.

Prognostic Significance of BMPR2 and SPARC Expression—To examine the clinical significance of BMPR2 in dormancy, we analyzed a Gene Expression Omnibus dataset and found that high BMPR2 expression predicts longer recurrence-free survival in prostate cancer patients (Fig. 5*A*) (28). However, BMPR2 expression did not correlate with presurgical prostate specific antigen, age, stage, or grade of the disease (supplemental Fig. S4, *A–F*, and Table S1). We also established a correlative gene signature from prostate cancer patients who had high BMPR2 expression in their primary tumor using The Cancer Genome Atlas (TCGA) database and found that this signature is highly enriched in patients who did not experience recurrent disease (Fig. 5*B*). In addition, we performed immunohistochemical staining of patient samples with bone metastasis and found that BMPR2 was significantly down-regulated in bone metastatic lesions compared with the primary tumor in these patients (Fig. 5, *C* and *D*), suggesting that decreased expression of BMPR2 is a key factor for metastatic growth in bone. We also observed that primary tumors of patients with bone metastasis expressed significantly lower levels of SPARC and BMPR2 compared with patients with localized disease (Fig. 5, *E–G*). Tumors from metastasis-free patients were significantly more positive for both SPARC and BMPR2 expression, whereas low SPARC, low BMPR2, or low SPARC and BMPR2 levels defined patients with bone metastatic disease. These results strongly support the notion that both SPARC and BMPR2 are crucial

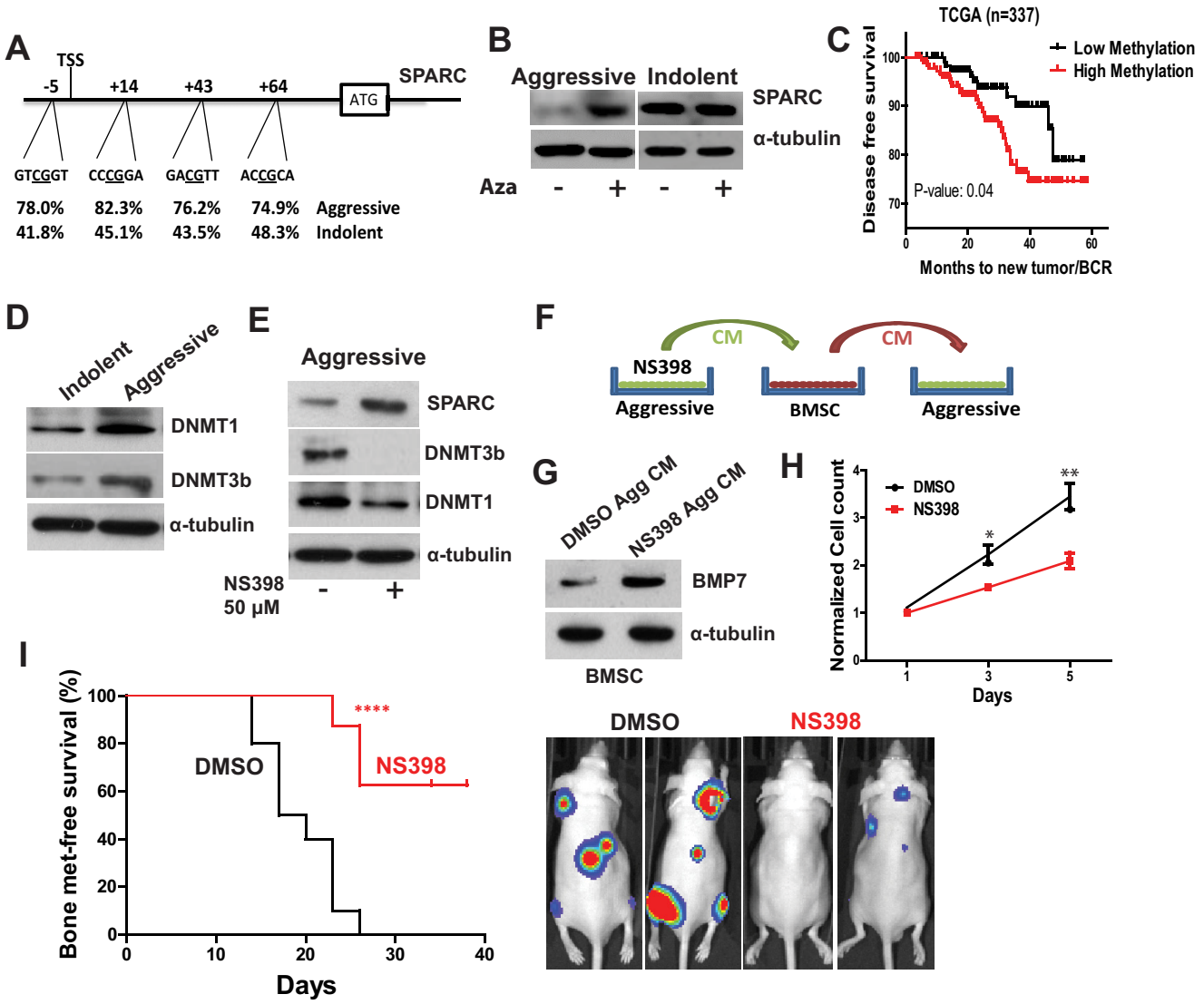

FIGURE 6. **SPARC is regulated by promoter methylation.** *A*, methylation of the SPARC promoter around the transcription start site (*TSS*) was examined for indolent and aggressive cells. *B*, aggressive and indolent cells were treated with 5-azacytidine (*Aza*, 50 nM), and SPARC expression was examined by Western blotting. *C*, prostate cancer patients in TCGA database were stratified based on the level of average SPARC promoter methylation, and new incidence or disease-free survival was examined for 5 years ($n = 337$). *BCR*; biochemical recurrence. *D*, DNMT1 and DNMT3b expression was examined in indolent and aggressive cells by Western blotting. *E*, aggressive cells were treated with NS398 (50 μM) for 24 h, and the expression of SPARC, DNMT1, and DNMT3b was examined by Western blotting. *F*, schematic of the collection of conditioned medium from aggressive cells treated with or without NS398, followed by treatment of BMSC. *G*, the expression of BMP7 was examined in BMSC after treatment with the indicated CM by Western blotting. *Agg*, aggressive. *H*, a cell proliferation assay was performed after treating aggressive cells with the CM outlined in *F*, and cell growth was examined on days 1, 3, and 5. A normalized cell count relative to day 1 is shown. *, $p < 0.05$; **, $p < 0.01$. *I*, aggressive cells were injected via the intracardiac route, and bone metastasis-free survival was examined in DMSO- or NS398-treated groups ($n = 10$/group). ****, $p < 0.0001$.

in dormancy and that the SPARC-BMP7-BMPR2 axis enhances dormant survival of cancer cells in bone.

The SPARC Gene Is Epigenetically Regulated in Aggressive Cells—The striking difference in the expression of SPARC between indolent and aggressive cells prompted us to test whether SPARC is epigenetically regulated during dormancy and recurrence. We therefore examined the methylation status of the SPARC promoter in indolent and aggressive cells. As shown in Fig. 6*A*, we found that several CpG islands in the promoter region of SPARC are significantly more methylated in aggressive cells compared with indolent cells. In addition, treatment with the demethylating agent 5-azacytidine reversed SPARC expression in aggressive cells but not in indolent cells (Fig. 6*B* and supplemental Fig. S5*A*). Furthermore, analysis of TCGA database for SPARC promoter methylation revealed that the SPARC promoter is highly methylated in prostate tumors compared with normal tissues (supplemental Fig. S5*B*). To examine the clinical relevance of SPARC promoter methylation, we stratified patients in TCGA database according to their SPARC methylation levels and found that low SPARC promoter methylation was significantly associated with increased disease-free survival in prostate cancer patients (Fig. 6*C*). In addition, we found that DNMT1 and DNMT3b, two dominant *de novo* DNA methylases that have also been known previously to regulate gene expression in tumor cells, were highly expressed in aggressive cells (Fig. 6*D*). Furthermore, the results of a clinical data analysis indicate that patients expressing high levels of DNMT1 and DNMT3b showed decreased

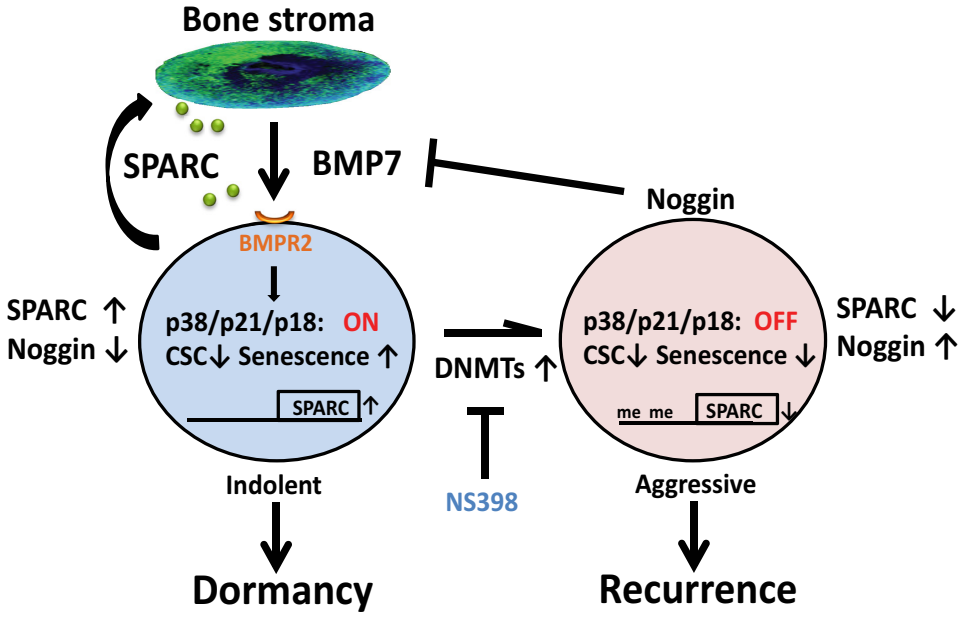

FIGURE 7. **Schematic illustrating how SPARC in the bone microenvironment induces dormancy.**

recurrence-free survival (supplemental Fig. S5, *C* and *D*). Interestingly, SPARC was shown previously to be down-regulated by the COX2 inhibitor NS398 via down-regulation of DNMT1 and DNMT3b in lung cancer cells (29). We found that NS398 treatment also reduced the expression of both DNMT1 and DNMT3b and increased SPARC expression in aggressive cells in a COX2-independent manner (Fig. 6*E* and supplemental Fig. S5, *E* and *F*).

To clarify the functional significance of this epigenetic control in dormancy, we first treated aggressive cells with NS398, followed by collecting CM, as illustrated in Fig. 6*F*. When BMSC were treated with this CM, BMP7 was significantly up-regulated (Fig. 6*G*). Furthermore, CM generated by treating BMSC with CM derived from NS398-treated aggressive cell was able to significantly lower the cell proliferation ability of aggressive cells (Fig. 6*H*). In addition, CM generated from azacytidine-treated aggressive cells was also able to up-regulate BMP7 in BMSC and decreased the cell proliferation ability of cancer cells (supplemental Fig. S5, *G* and *H*). These results suggest that reversal of SPARC methylation offers a window of therapeutic opportunity for recurrent disease by enhancing inhibitory signaling from the bone stroma. We transplanted aggressive cells intracardially and treated the animals with NS398. As shown in Fig. 6*I*, treatment with NS398 significantly suppressed tumor incidence in the bones of these animals. These results suggest that reversal of SPARC promoter methylation induces dormant survival of cancer cells in bone by educating stromal cells for an inhibitory response via BMP7 secretion.

Discussion

Prostate tumor cells are known to often disseminate at the very early stage of tumorigenesis, and ~90% of metastatic disease is related to bone, suggesting that tumor cells reside in bone for a prolonged period of time before growing as overt metastases (30). The well established tumor-supportive niche provided by the bone environment makes it one of the sanctuary sites for dormant survival of disseminated tumor cells, as evidenced by the isolation of dormant disseminated tumor cells from the bone marrow of a patient with a history of prostate cancer (5). In our study, by injecting CSC in the tibial bone of mice, we isolated a pair of cell lines (aggressive and indolent cells) that mimic the phenomenon of metastatic dormancy in bone. To our knowledge, this is the first established pair of cell lines that mimics the phenotype of dormant and aggressive growth in bone *in vivo*. Importantly, the recurrence assay shown in Fig. 2*D* verified the reversibility of dormant tumor cells, and therefore our model can provide a valuable tool to study dormancy and recurrence. The indolent cells activated the p38 MAPK pathway and its downstream cell cycle inhibitors only when co-cultured with bone stromal cells, which illustrates the importance of cancer-stroma cross-talk and p38 activation in the maintenance of the dormant niche in bone. Our results also showed that this cross-talk was mediated via SPARC secreted by indolent cells and that SPARC stimulated the paracrine inhibitory response through the BMP7-BMPR2 axis. Importantly, SPARC was epigenetically silenced by promoter methylation in aggressive cells, and treatment with NS398 reversed SPARC methylation and enhanced dormant survival in bone by down-regulating DNA methylase enzymes. Therefore, this potential therapeutic strategy may keep cancer cells in perpetual dormancy. Fig. 7 illustrates how SPARC induces dormancy in the bone microenvironment.

SPARC is an extracellular matrix-associated protein known for its oncogenic and tumor-suppressive roles (31). SPARC has been documented for its role in the formation of extracellular matrix and mineralization of bone (19, 20, 32, 33). SPARC plays a significant role in tissue remodeling, maintaining cell matrix integrity and interaction and collagen fiber assembly (34). Stroma- as well as tumor-secreted SPARC is known to affect tumor growth in cell type- and context-dependent manners by regu-

lating cell proliferation, adhesion, migration, invasion, and angiogenesis (25, 35–38). In prostate cancer, apparent conflicting results regarding SPARC expression in both clinical and experimental studies suggest that the role of SPARC is even more complex. The studies that involved unbiased screening by gene expression analysis have shown SPARC expression to be correlated with high-grade, androgen-resistant, and metastatic disease. In addition, SPARC was shown previously to attract prostate cancer cells into bone by promoting a migratory and invasive phenotype (39–42). Indeed, a moderate level of expression of SPARC was observed in bone metastases of prostate cancer patients by immunohistochemistry (43). On the contrary, several lines of evidence indicate tumor-suppressive roles of SPARC in prostate cancer (44–47). Wong *et al.* (47) found a significantly diminished SPARC level in patients with metastatic disease. Similarly, Kwabi-Addo *et al.* (48) have shown that SPARC is silenced by promoter methylation in African-American patients who often develop aggressive prostate cancer with 2-fold higher mortality rate than Caucasian American patients (48). The apparent disagreement between these studies may be due to multiple factors, including expression profiling of the primary tumor, batch variability, and lack of clinical data for the site of metastasis. Moreover, the controversy of the pathological role of SPARC in prostate cancer was further affirmed in two separate studies that utilized the SPARC knockout and transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse models. Although Said *et al.* (25) found an inhibitory role of SPARC in tumor and metastatic growth, another study did not identify any role of SPARC in tumor progression and metastasis (49). The discrepancy in the pathological outcome evident in these studies might be due to the differences in genetic background of the mice used in the study. Similarly, controversy over the role of SPARC was also evident in carcinoma of breast and skin, where both oncogenic and tumor-suppressive roles were observed in multiple *in vitro*, *in vivo*, and clinical studies (50). On the other hand, the tumor- and metastasis-suppressive role of SPARC has been well documented for gastrointestinal, ovarian, pancreatic, and colon cancers, whereas the oncogenic role was verified in glioma (38, 51–55). Therefore, the role of SPARC in tumor progression appears to be complex and specific to the tissue type and stages of the disease. Our data suggest the new paradigm that SPARC is highly expressed by dormant cancer cells residing in bone, which demonstrates that the role of SPARC is contextually and spatiotemporally regulated. We found that bone stromal cells specifically responded to tumor-induced SPARC by secretion of tumor-inhibitory BMP7, which, in turn, activated the p38 MAPK pathway via BMPR2 in cancer cells. Moreover, BMP7 also decreased stem cell population and enhanced reversible senescence of cancer cells. On the other hand, disruption of BMP7-BMPR2 signaling axis by Noggin, a potent competitive inhibitor of BMPs, rescued the inhibitory effect of BMP7 in the bone microenvironment. Therefore, the precise role of SPARC is greatly dependent on the tumor-niche proteome profile and crosstalk with the microenvironment. It is conceivable that education of tumor stroma by SPARC and explicit stromal response at various stages of tumor progression greatly dictates the functional fate of SPARC.

Multiple types of cancer cells are known to interact and communicate with cells in the bone marrow microenvironment through secretion of cytoactive molecules. McCabe *et al.* (56) observed that SPARC suppressed osteoclast differentiation, a key step during bone tumor growth, wherein SPARC knockout mice showed increased osteolysis after intraosseous implantation of RM1 murine prostate cancer cells. Similarly, when cancer cells were grown in the bone matrices generated *in vitro* using SPARC knockout osteoblasts, the bone growth of PC3 cells was greatly enhanced (57). Furthermore, the bone metastasis-suppressive function of SPARC was reported previously for breast cancer by utilizing an *in vivo* systemic inoculation model (51). Based on our results of SPARC-mediated cross-talk with BMSC, it is plausible that inhibition of cancer growth in these models involves inhibitory paracrine factor(s), such as BMP7, from the stroma. Interestingly, a previous study has shown that tumor-stroma interaction in the bone elevates SPARC expression in the bone microenvironment, followed by proteolytic cleavage by the stromal collagenase cathepsin K, although the specific role of SPARC fragments in bone metastasis was not examined (58). It is noteworthy that opposing biological roles of SPARC fragments have been identified previously (50). Therefore, the differences in the protease profile of the microenvironment may potentially dictate the functional fate of the cleaved fragments, which warrants further studies to identify the functions of individual peptides during the pathological stages of bone metastasis.

Bone stromal cells were reported previously to induce dormancy of cancer cells either through exosome-mediated secretion of inhibitory microRNAs or through secretion of stromal proteins such as TGFβ2 and BMP7(18, 59). BMP7 is known to affect invasion and migration by inhibiting epithelial-to-mesenchymal transition of cancer cells (60, 61). We showed previously that BMP7 released by the bone stroma decreased stemness and promoted reversible senescence of cancer cells via signaling through BMPR2. Intriguingly, we found in this study that SPARC elevates BMP7 expression from BMSC, which led to senescence of indolent cells. In addition, the CM from SPARC-treated BMSC also promoted the senescence phenotype and reduced the stem cell population in cancer cells. Therefore, indolent cells maintain a dormant state in the bone microenvironment through activation of inhibitory signaling mediated by the BMP7-BMPR2 axis. Notably, aggressive cells expressed a high level of Noggin, and therefore it is highly probable that the elevated Noggin expression in cancer cell or the bone microenvironment disrupts BMP7-BMPR2 signaling, rescues cancer cells from the dormant state, and triggers the onset of recurrent disease.

Our finding shows that BMPR2 expression plays a key role in dormant survival of cancer cells in bone. The major molecular phenotype of dormancy, reduction in stemness, was not evident when BMPR2 expression was knocked down in PC3mm cells. In addition, the BMPR2 level in the tumor of a patient negatively correlated with the status of recurrent disease, and the BMPR2-correlative signature was highly enriched in patients who did not experience recurrent disease. In support of our finding, BMPR2 expression was reported previously to be lost in aggressive disease of bladder and colon cancers (62, 63).

Importantly, our immunohistochemical analysis also revealed decreased expression of BMPR2 in bone metastatic primary tumors as well as bone lesions compared with the primary tumor without metastasis. Therefore, it is conceivable that decreased BMPR2 expression or downstream signaling activation may lead to a conducive environment in bone for recurrent tumor growth. These results further underline the potential utility of BMPR2 and its downstream proteins as biomarkers for patient prognosis.

The differential methylation status of the SPARC promoter in indolent and aggressive cells suggests that environmental stress reprograms tumor cells for dormant survival via epigenetic modification. Indeed, our results revealed that reversal of SPARC promoter methylation in Aggressive cells either by 5-azacytidine or NS398 treatment enhanced SPARC expression and induced the inhibitory signal from stromal cells. Methylation of promoters of prominent tumor-suppressive genes has been known to enhance aggressive growth at distant sites (64). Sosa *et al.* (13, 14) have shown that NR2F1, an orphan nuclear receptor of RA signaling, rendered the dormancy phenotype *in vivo* by activating global repressive chromatin marks in cancer cells. Interestingly, NR2F1 binds and regulate the SPARC promoter in head and neck squamous cell carcinoma, suggesting a possibility that selection of an indolent clone is dependent on an epigenetic master regulator that changes the expression of prominent gene promoters (13). The increased expression of *de novo* methylase genes DNMT1 and DNMT3b in aggressive cells further supports the notion that epigenetic silencing of the SPARC promoter by methylation may be a potential key for recurrent growth in bone (Fig. 7). In support of this notion, patients with low promoter methylation status of SPARC showed prolonged disease-free survival. Furthermore, NS398 induced SPARC via down-regulation of DNMT1 and DNMT3b, which is in agreement with a previous study showing a DNMT-dependent increase of SPARC in A549 lung carcinoma cells by NS398 (29). In addition, NS398 has been studied previously for its effect on limiting cell proliferation, angiogenesis, invasion, and metastasis of multiple cancer types (65–67). It should be noted that NS398 was also shown previously to significantly inhibit bone metastasis of breast cancer cells by suppressing TGF-β dependent activation of COX-2. It is conceivable to induce SPARC by NS398 to maintain the dormant state of cancer cells in bone (68). To this end, we transplanted aggressive cells and found that treatment with NS398 significantly inhibited metastatic growth in bone. This finding shows a potential use of NS398 to maintain cancer cells in the dormant state and offers therapeutic windows to treat bone recurrent disease.

Experimental Procedures

Cell Culture—Indolent and aggressive cell lines were isolated from tibial bone of nude mice after injection of PC3mm cancer stem like cells as described in supplemental Fig. S1*A*. The PC3mm cell line was provided by I. J. Fidler (University of Texas MD Anderson Cancer Center, Houston, TX). DU145 was obtained from the American Type Culture Collection. ALVA41[e] was provided by W. Rosner (Columbia University, New York, NY). LNCaP cells were obtained from the University of Texas MD Anderson Cancer Center. hBMSCs (donors 7075 and 7083) were obtained from the Texas A&M Institute for Regenerative Medicine. hBMSCs were maintained in minimum essential medium with 20% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. Other cells were grown in RPMI 1640 medium with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin and incubated at 37 °C in a 5% CO_2 atmosphere. The PC3mm cell line was transduced with a lentiviral vector carrying the firefly luciferase gene for bioluminescence tracking. Recombinant human SPARC and BMP7 were purchased from ProSpec. For transwell co-culture, 10^4 cancer cells were seeded on a 24-well plate, and a culture insert with 0.4-μm pore size (Corning) was placed on top of each well, followed by seeding the upper chamber with 10^5 BMSCs.

Preparation of Conditioned Medium—For generating BMSC + SP CM, hBMSC were treated with 0.2 μg/ml recombinant SPARC and incubated for 24 h, and the medium was replaced with serum-free RPMI medium. After 24 h of incubation with serum-free RPMI medium, the CM was collected and added to cancer cells at a ratio of 50:50 with 10% RPMI. For supplemental Fig. S4*D*, aggressive cells were treated with 0.5 μM 5-azacytidine or vehicle (1:1, acetic acid:water) for 4 days, followed by replacing the medium with serum-free RPMI medium. The cells were then incubated for 24 h, and CM was collected. This CM was further added to hBMSC and incubated for 24 h, followed by changing of the medium to serum-free RPMI. After 24 h, CM was collected and added to aggressive cells.

Isolation of CSCs—CSCs were isolated by magnetic bead sorting using a magnetic-assisted cell sorting (MACS) separator (Miltenyi Biotec) as described previously (18). PC3mm cells were incubated with the following specific antibodies: anti-CD24-biotin (STEMCELL Technologies), anti-CD44-APC (BioLegend), and anti-CD133-biotin (Miltenyi Biotec). $CD24^{low}/CD44^{high}/CD133^{high}$ cells were then enriched by using a magnetic-assisted cell sorting magnet and MS columns (Miltenyi Biotec). All magnetic-assisted cell sorting procedures were performed according to the instructions of the manufacturer.

FACS—Prostate cancer cells were treated with CM for 48 h. CM was replaced after 24 h. Cells were then collected after 48 h, washed twice in PBS, incubated with CD24-FITC, CD44-APC and CD133-PE for 20 min, and analyzed for CSC positive population ($CD24^{low}/CD44^{high}/CD133^{high}$) in a BD Accuri instrument.

Western Blotting—The cells were lysed and analyzed by immunoblotting using antibodies specific for the following proteins: p21, p18, α-tubulin, and GAPDH (Cell Signaling Technology); SPARC (R&D Systems); DNMT3b, Noggin, and BMP7 (Abcam); and DNMT1 (Genetex).

Sphere-forming Assay—Cancer cells were plated (200 cells/well) in 96-well ultra-low attachment plates (Corning) with DMEM/F12 supplemented with 2% B27 (Invitrogen), 20 ng/ml EGF (Sigma-Aldrich), and 4 μg/ml insulin (Sigma-Aldrich). They were then incubated with CM generated with or without treatment of BMSC with recombinant SPARC. The number of prostaspheres was counted, and data are represented as the mean ± S.E.

Proliferation Assay—Cells were seeded into 96-well plates (500 cells/well) in regular growth medium. The cells were then cultured overnight, followed by treating them with conditioned medium. Cell viability was measured by (3-(4,5-dimethylthiazol-2 yl)-5-(3-carboxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay according to the recommendations of the manufacturer (Promega).

Animal Experiments—All animal experiments were done in accordance with a protocol approved by the Wake Forest Institutional Animal Care and Use Committee. Athymic nude mice (Harlan) 4–5 and 7–8 weeks of age were used for the xenograft experiment. For isolating indolent and aggressive cells, 4000 PC3mm CSCs labeled with luciferase were injected into the tibial bone of nude mice. After 6 weeks, tibial bones with aggressive or indolent tumor growth were flushed with PBS, followed by selection of the cells with puromycin. For verification of differential growth, 10^4 indolent or aggressive cells were injected into the tibial bone. For bone metastasis-free survival experiments, 10^5 CSCs isolated from indolent or aggressive cells were injected into the left cardiac ventricles of mice. For the recurrence assay, 200 μg/kg SPARC or vehicle alone was subsequently injected every 2 days into the tail vein. For NS398 treatment, 10^6 aggressive cells were injected into the left cardiac ventricle, and mice were treated with either DMSO or NS398 (20 mg/kg) every 2 days until day 40. The progression of cell growth and development of metastases was monitored by bioluminescence imaging.

Wound Healing Assay—Cells were grown until confluence, and cells were scratched by a 1-ml pipette tip. The migration of cells was monitored under the microscope after 9, 12, 24, and 48 h. The percentage of wound healing was counted in three different fields for each cell line.

Invasion Assay—Cell culture inserts with a microporous membrane were coated with Matrigel (BD Biosciences), followed by seeding 10^5 cancer cells. RPMI medium containing 20% fetal bovine serum was added to the bottom chamber. The cells were then incubated for 24 h at 37 °C. The upper chamber was removed, and the cells in the bottom chambers were stained with tetrazolium dye and counted under a microscope.

Bisulphite Sequencing—Genomic DNA was isolated using a cell and soft tissue DNA isolation kit (Zymo Research). The methylation of the CpG island was examined by bisulphite sequencing (Epigendx).

Quantitative RT-PCR Analysis—Total RNA was isolated from the cells and reverse-transcribed. The cDNA was then amplified with a pair of forward and reverse primers to validate the results of the microarray. The thermal cycling conditions consisted of an initial denaturation step at 95 °C for 1 min, followed by 35 cycles of PCR using the following profile: 94 °C for 30s, 58 °C for 30 s, and 72 °C for 30 s.

Statistical Analysis—Results are reported as mean ± S.E. For *in vitro* experiments, Student's *t* test or one-way analysis of variance was applied. For *in vivo* experiments, group comparisons were performed using nonparametric Mann-Whitney test or unpaired Student's *t* test. Kaplan-Meier curve comparison was performed with a log-lank test.

Senescence-associated β-gal Staining—Cells were treated with or without SPARC-treated BMSC CM as indicated in the figure legends, and a senescence-associated β-gal assay was performed using the senescence-associated β-gal staining kit (Cell Signaling Technology) according to the instructions of the manufacturer.

Gene Set Enrichment Analysis—The Gene MatriX file (.gmx) was generated by combining the top 378 genes that were significantly correlated ($R^2 > 0.5$) to BMPR2 in TCGA database with 502 prostate cancer patients. The Gene Cluster Text file (.gct) was generated from Taylor's cohort (GSE21034) by separating prostate cancer patients based on their status of recurrence. Patients who did not experience recurrence for at least 5 years were placed in the "no recurrence" group ($n = 32$), whereas patients who experienced recurrence before 5 years were placed in the "recurrence" group ($n = 34$). Similarly, the Categorical class file (.cls) was also generated based on the recurrence status of each patient. The number of permutations was set to 1000, and we used GPL10264 as the chip platform.

Immunohistochemistry—Primary prostate cancer tissue microarrays were obtained from US Biomax, Inc. (PRT195 and PR242b). The tissue microarray generated from bone metastatic lesions was obtained from Tirstar Inc. (79562475). Formaldehyde-fixed and paraffin-embedded human prostate tissue specimens were obtained from the surgical pathology archives of the Akita Red Cross Hospital (Akita, Japan) and Iwate Medical School (Iwate, Japan). The sections were baked at 60 °C for 1 h, deparaffinized in xylene, and rehydrated and fixed in 10% neutral buffered formalin. Immunohistochemical staining of histological sections was performed according to a protocol published previously (18) using the EnVision Plus system (Dako) and antibodies specific to BMPR2 (Abcam) and SPARC (R&D Systems).

Cell Labeling with DiD Dye—Cells were stained with DiD dye (Molecular Probes, Eugene, OR; catalog no. V22887) according to the instructions of the manufacturer. Briefly, cells (1×10^6 cells/ml) were incubated with DiD dye (0.5 μM) in serum-free medium at 37 °C for 40 min, washed with serum-free medium three times, resuspended in PBS, and analyzed for staining by FACS (BD Biosciences, Accuri).

Author Contributions—S. S. conducted most of the experiments, analyzed the results, and wrote most of the manuscript. F. X. conducted intracardiac injections. Y. L. conducted the bioinformatics analysis. K. Wu conducted tail vein injections. Y. S. conducted intratibial injections. R. P. prepared conditioned medium from BMSC. N. S., H. K. L., and K. C. B. contributed to immunohistochemistry staining and Western blotting. K. Watabe conceived the idea for the project and contributed to editing the manuscript.

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